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Electrochemical immunosensor for tumor necrosis factor-alpha detection in undiluted serum

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Abstract

An immunosensor for the sensitive detection and estimation of tumor necrosis factor-alpha (TNF- α) in undiluted serum has been developed via an electrochemical enzyme-linked immunosorbent assay (ELISA) process. Electrochemical sensing was performed using a TNF- α specific monoclonal antibody modified self-assembled monolayer of dithiobis(succinimidyl propionate) on a comb-shaped gold electrode microarray. After anti-TNF- α antibody binding, unreacted active groups of DTSP were blocked using ethanol amine (EA) and nonspecific binding was prevented using phosphate buffer based starting block T20 (SB). Sensitive and disposable SB-EA-anti-TNF- α /DTSP/Au electrodes were exposed to solutions with different TNF- α concentrations for 20 min in undiluted serum. Conversion of 4-aminophenyl phosphate to 4-aminophenol and its electrochemical oxidation was utilized for indirect estimation of TNF- α . Results for SB-anti-TNF- α /DTSP/Au electrodes indicate that the sensors can be used for the sensitive estimation of TNF- α in undiluted serum in the range 500 pg/ml to 100 ng/ml with a detection limit of 60 pg/ml and sensitivity of 0.46 (ng/ml)⁻¹. Negligible interference from serum and other biomarker proteins was observed. The described electrochemical ELISA is much faster than conventional ELISA and can be applied for sensing of a range of analytes in real patient samples.

Keywords: Dithiobis(succinimidyl propionate); electrochemical ELISA; serum; TNF- α ; immunoassay

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1. Introduction

With an ever increasing demand for improved healthcare, informative biomolecules known as biomarkers are gaining much attention for accurate and faster diagnosis and prognosis of a specific disease.[1, 2] These can be found at the early stage of diseases and quantification of these biomarkers from blood, urine or other tissues can provide vital information regarding patient health.[3] Thus, detection and estimation of their level accurately and sensitively is critical for early stage identification and proper recovery. Several approaches and protocols for their detection, such as optical (fluorescence, chemiluminescence, UV-visible), biophotonic, electrochemical sensors, electro-chemiluminescence, etc. have been reported in literature.[3-5] Among these, optical methods such as ELISA, fluorescence and bio-photonic have provided high quality data and are most often been employed for detection. However, such optical methods require complex equipment and trained personnel for operation; thus the development of alternative methods that give high quality data with simple procedures is desired.[4, 6] Owing to their accuracy, higher sensitivity, portability, low cost and simplicity, electrochemical immunosensors have recently gained significant attention.[1, 3, 4, 7, 8] Among electrochemical immunosensors, electrochemical ELISA, which uses the selectivity of an antigen–antibody interaction on an electrochemical platform, is seen as one of the most promising methods. Electrochemical ELISA has the added advantage of not being affected by particles, chromophores, and fluorophores that might be present in a sample and which might cause interference with optical detection.[9-11] However, a simpler method for developing a convenient, stable and sensitive electrochemical ELISA still requires significant research.

In the development of an immunosensor, the immobilization of a capturing molecule, such as an antibody, onto a surface is crucial and can determine the stability of the sensor.[10-14] In attempts to immobilize and stabilize capturing biomolecules, a number of approaches such as the use of direct physical adsorption, self-assembled monolayers (SAM), polymer membranes, etc.

have been investigated.[1, 10, 11, 15] Among the covalent immobilization methods, use of thiol SAMs has shown a number of advantages such as an easy and efficient immobilization with uniform surface coverage. However, most of the SAMs require either activation or further modification before immobilization of the bioreceptor, which might disturb the integrity of the biomolecule.[16] Recently, SAMs based on dithiobissuccinimidyl propionate (DTSP) have shown that they can be used for facile covalent binding of biomolecules under very mild conditions, thus preserving their activity.[17-19] For successful development, a desirable biosensor should have capability to identify the desired biomarker in an unbiased manner in complex samples (e.g. serum) with various potentially interfering materials. Eliminating serum background is critical for biosensor development as serum contains high levels of cells, proteins, electrolytes, lipids, etc. which may interfere with the detection signal and may give rise to false positive signals in real samples.[4] For point-of-care (POC) applications, where both sample collection and the test are done on site, the use of blood or serum as a sample is unavoidable. Researchers have described various methods to reduce the serum background interference.[4, 20, 21] Among these, the use of serum dilution before testing has proven to be quite an effective method; however it also causes dilution of the target protein, thus lowering its accurate detection.

In the present work, an electrochemical ELISA based system has been developed by solving various issues involved in their development. DTSP has been used to immobilize antibody on comb-shaped gold micro array electrodes. A phosphate buffer saline / tween20 based starting blocker (SB) has been utilized for preventing false signals from detection of target tumor necrosis factor-alpha (TNF- α) protein in undiluted serum. SB contains a proprietary protein that provides exceptional performance and broad compatibility with ELISA assays. Furthermore, alkaline phosphatase that can biocatalyze 4-aminophenyl phosphate (4-APP), resulting in enhanced redox signal by enriching the catalysed product on the working electrode during sandwich assay, has been employed to get higher response. During the immunoassay, increasing TNF- α concentration causes

increased antigen binding with immobilized antibodies on the sensor surface, which upon interaction with a second antibody and ALP-streptavidin results in higher conversion of 4-APP to 4-amino phenol. On electrochemical oxidation, this releases two electrons to produce quinonimide and is therefore detected on the surface. Figure 1b shows the schematic steps of the immunoassay and detection process. The chosen TNF- α protein is a cytokine serving as potent protein biomarker for various physiological and pathological processes such as apoptotic cell death, fever and cachexia. It is also known to be involved in immune system regulation and in systemic inflammation.[3] In healthy individuals, cytokine levels, like TNF- α , usually exist from pg/ml to ng/ml levels, however in pathological conditions such as rheumatoid arthritis (RA), its level rises by ten to hundred times causing local cell apoptosis, thus necessitating rapid detection method for early disease diagnosis and prognosis. Its elevated levels to ng/ml in serum has also shown to be associated with a wide range of diseases, such as systemic erythema nodosum leprosum, Crohn's disease, endotoxic shock, HIV infection, stroke, neonatal listeriosis, severe meningococemia and osteosarcoma [1, 3, 7, 15, 18, 22, 23], thus making it an important biomarker necessitating the development of immunosensors for its sensitive and selective detection. Current methods of detecting TNF- α include optical enzyme-linked immunosorbent assay (ELISA) along with chemiluminescence, radio-immunoassays, mass spectrometry/laser desorption, flow cytometry, enzyme-linked immunosorbent spot assay, and immuno-PCR.[3-5, 15, 24] Among these commercially available products, ELISA is quite sensitive and mainly employed; however it suffers from relatively high background signal and long procedure, bulky instrumentation and requirement of trained personnel. Other methods are also expensive, laborious and suffer from similar problems. In the present work attempt, a self-assembled monolayer based electrochemical ELISA for protein biomarker detection in undiluted serum has been developed.

2. Materials and Methods

2.1 Reagents

TNF- α , primary antibody and biotin-conjugated secondary antibody were procured from Biolegend (USA); streptavidin-conjugated alkaline phosphate (ALP) solution from Affymetrix UK Ltd; 4-aminophenyl phosphate monosodium salt (4-APP) from Santa Cruz Biotechnology (USA); starting block PBST20, starting block TBST20 and dithiobis (succinimidyl propionate) (DTSP) from Fisher Scientific (UK); FemtoTBS from Gbioscience (UK); sodium borohydride and dimethyl sulfoxide (DMSO) from Sigma (UK). All other chemicals were of analytical grade and were used without further purification.

2.2 Gold electrode fabrication

Comb-shaped gold micro-electrode arrays were created using lithographic and micro-fabrication techniques on silicon wafers with a thick layer of thermally grown oxide layer as described in an earlier report.[18] Comb fingers of 5 μm thickness and 3200 μm length, spaced at 25 μm were created over the length of 5500 μm . Counter and *pseudo* reference electrodes of gold around the working comb shape electrode were deposited on the chip in the same manner. The fabricated electrodes were pre-cleaned with isopropyl alcohol, acetone and with copious amounts of de-ionized milli-Q water (Millipore, UK) followed by treating for 30 min using UV-ozone (ProCleaner, BioForce Nanosciences, USA) before use.

2.3 DTSP SAM formation and immobilization of anti-TNF- α antibodies

For DTSP SAM formation, 1 ml of 2 mg/ml solution of DTSP in DMSO was prepared and reduced with 5 μl of sodium borohydride (10 mg/ml in H_2O) for 10 min at room temperature. Each gold electrode was then incubated for 1 h with 100 μl of DTSP solution at room temperature ($\sim 22^\circ\text{C}$). After SAM formation, unbound DTSP molecules were removed by washing the electrodes with DMSO, acetone and milli-Q water, followed by air blow drying. A solution chamber covering

the SAM-modified electrodes was created by fixing a laser cut double-sided tape (Figure 1). To make the chamber, liner on one side of tape was peeled and pasted on electrode making a very thin chamber, capable of holding 50 μ l solution covering the entire chamber. Figure 1 shows the picture of the sensor chip with and without double-sided tape chamber along with a schematic of the SB-ethanol amine-anti-TNF- α /DTSP/Au fabrication. The DTSP modified electrodes were then incubated with 10 μ g/ml TNF- α antibody solution in PBS for 90 min at 37 $^{\circ}$ C. During incubation, antibodies were covalently attached to the DTSP surface via nucleophilic attack of amino groups of antibodies to reactive succinimidyl groups on DTSP. The prepared anti-TNF- α /DSP/Au electrode was washed thoroughly with PBS followed by incubation with 1% ethanol amine (EA) in PBS for 10 min and again washed with PBS to block any remaining active groups of DTSP. Electrodes were then incubated with starting block PBST20 (SB) for 30 min to block empty places on the electrode surface and to prevent any non-specific adsorption. The prepared electrodes were stored at 4 $^{\circ}$ C when not in use and were characterized using optical and electrochemical techniques during experimentation.

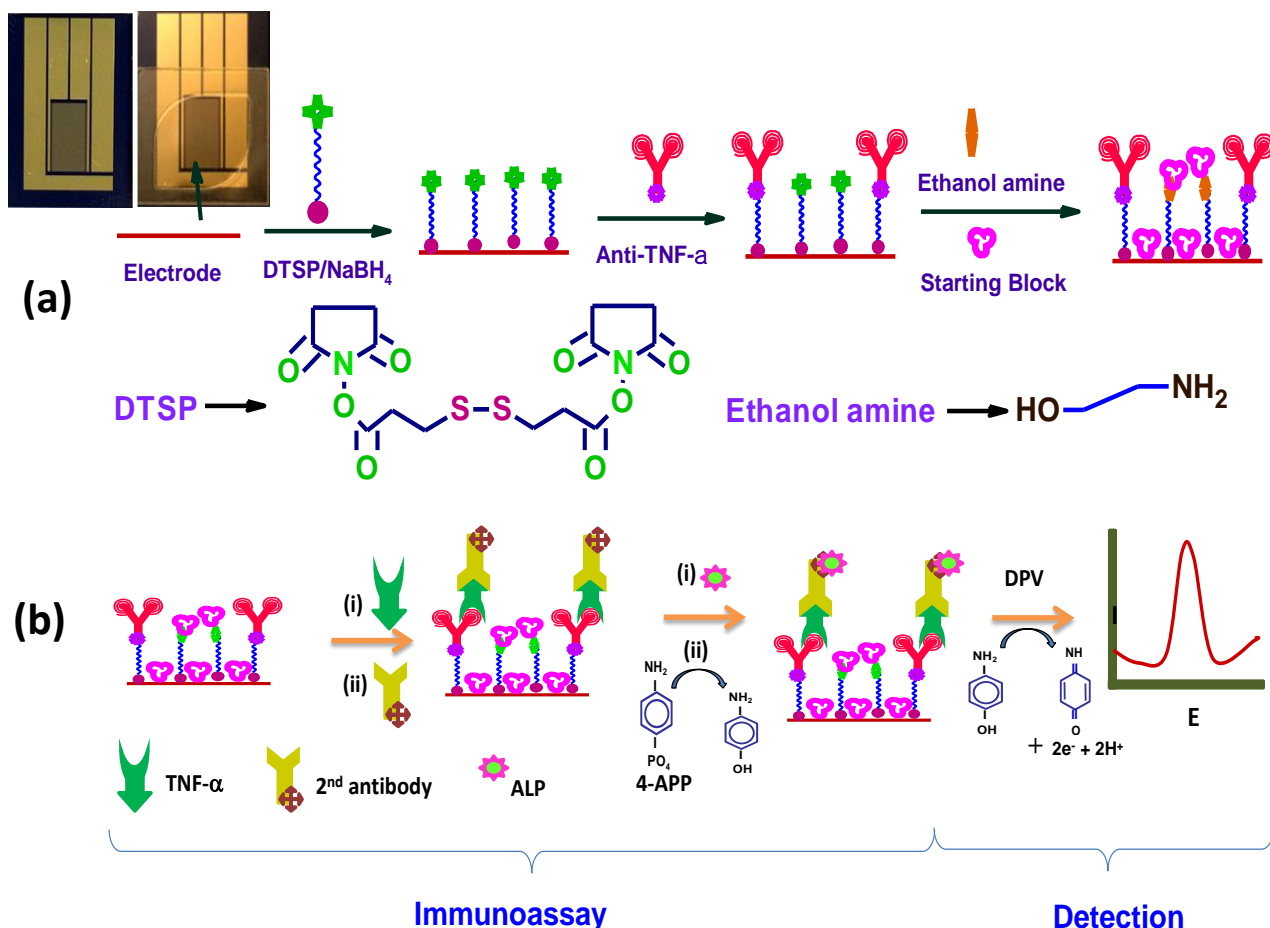


Figure 1. (a) Schematic of the SB-EA-anti-TNF- α /DTSP/Au fabrication. (b) Schematic for immunoassay and testing.

2.4 Immunoassay process sequence

The electrochemical immunoassay started with incubation of the sensor chip with 50 μ l of the desired concentration of TNF- α in undiluted serum, where anti-TNF- α antibodies bound on sensor surface capture TNF- α in the sample. On completion of optimized 20 min of incubation, the sensor chip was rinsed thoroughly using 1x Femto TBST washing buffer. After the antigen binding step, 50 μ l of 10 μ g/ml secondary anti-TNF- α with biotin tag solution in starting block TBST20 was poured on the sensor and incubated for another 20 min followed by thorough washing with 1x Femto TBST. The secondary anti-TNF- α modified sensor was then allowed to interact for 20 min with streptavidin-alkaline phosphatase solution in starting block TBST20 (50 μ l of 10 μ g/ml)

followed by washing again with 1x Femto TBST. In a final incubation, the sensor chip was incubated with 50 μ l of 2 mg/ml 4-APP in deoxygenated tris-HCl buffer (100 mM, pH 9) containing 4 mg/ml $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ for 20 min followed by differential pulse voltammetric (DPV) signal recording. To record the DPV signal, conditions were initially taken from Bettazi et al. [23]; however to avoid oxygen interference and to record optimum oxidation signal of 4-amino phenol to quinonimide at around 0.2 V, a potential range of -0.2 to 0.4 V was selected. Also, the optimum response was observed when an amplitude of 25 mV, step potential of 10 mV, and interval time of 0.1 s were used, which were thus utilized in the present study. DPV measurement was recorded using an μ Autolab III / FRA2 potentiostat/galvanostat (Metrohm Autolab, Netherlands) controlled by General Purpose Electrochemical System (GPES) version 4.9 software. Undiluted serum was used as a control to monitor the effect of serum proteins on the measurement. All experiments for TNF- α detection were carried out at least in triplicate using separate electrodes and the standard deviations were represented via error bars. Figure 1b shows the schematic of immunoassay and testing.

3. Results and discussions

3.1 Chip fabrication and characterization.

Figure 2 shows the optical image of the prepared chip and its characterization using cyclic voltammetry and electrochemical impedance spectroscopy in 10 mM PBS pH 7.4 containing 5 mM $\text{FeCN}_6^{3-/4-}$. It is clear from Figure 2a that the prepared chip exhibits sharp oxidation and reduction peaks with equal current of around 155 μ A. Optical image in the inset shows the reference, counter and two working comb shaped micro electrodes array arranged in an interdigitated manner. Figure 2b shows a Nyquist plot for the cleaned blank chip in a three-electrodes configuration; good conductivity with low charge transfer resistance of around 141 Ω is observed. The slight variation in the diffusion curve for Warburg impedance region may be attributed to the effect caused by

exposed titanium layer under each gold comb finger. Results thus indicate the preparation of gold sensor chips. Also, chips prepared in batch showed almost the same response, with variations of less than 1%.

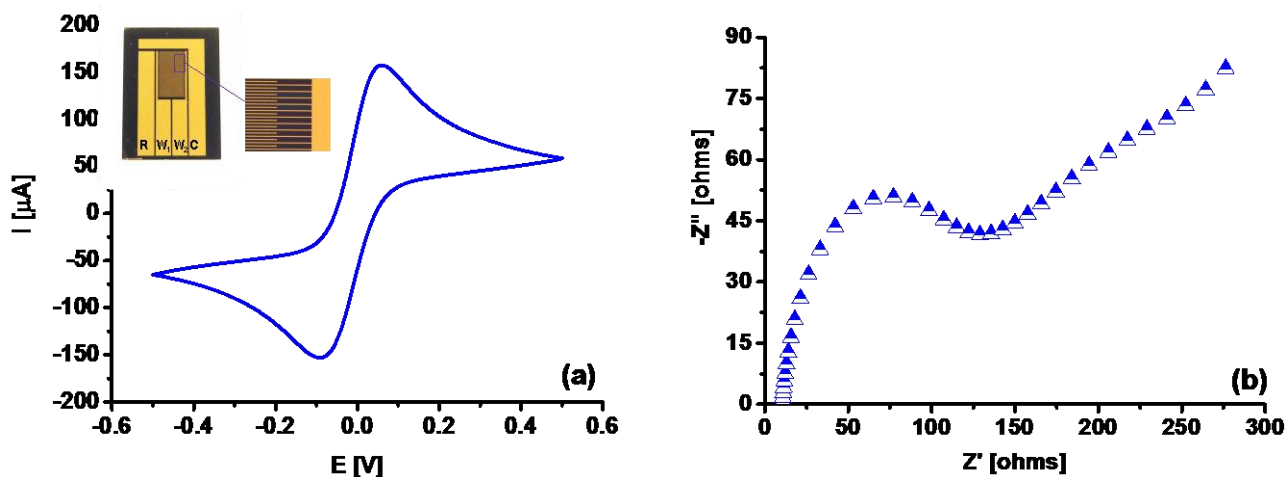


Figure 2 (a) Cyclic voltammetric and (b) electrochemical impedance scan for clean blank chip in 10 mM PBS pH 7.4 containing 5 mM $\text{FeCN}_6^{3-/4-}$. Inset in (a) shows the optical image of the prepared chip.

3.2 Atomic force microscopic imaging and contact angle measurements

Figure 3a-b shows atomic force microscopic (AFM) images of blank gold and EA-anti-TNF- α /DTSP/Au taken in tapping mode using a 10 nm AFM tip. The observed change of granular morphology for the blank gold surface (Figure 3a) to smoother and globular morphology for anti-TNF- α /DTSP/Au (Figure 3b) suggests the successful immobilization of anti-TNF- α onto the gold surface. Furthermore, contact angle measurements were carried out for the blank Au surface (Figure 3c), DTSP/Au (Figure 3d) and EA-anti-TNF- α /DTSP/Au (Figure 3e) to characterize anti-TNF- α immobilization. Contact angle measurements were performed using an in-house built optical angle measurement system. The electrodes were placed on the stage and a 10 μl drop of water was dispensed on the electrode with the dispensing system. The wetting of surface was then captured using a Nikon p520 camera and contact angle was measured using an on-screen protractor. In

Figure 3c-e increase in contact angle from 25° for the blank gold surface to 51° for DTSP/Au indicate the successful SAM formation. A decrease in contact angle value to 42° after anti-TNF- α binding confirm the immobilization of antibody; decrease in contact angle after anti-TNF- α binding might be attributed to the hydrophilic nature of antibody.

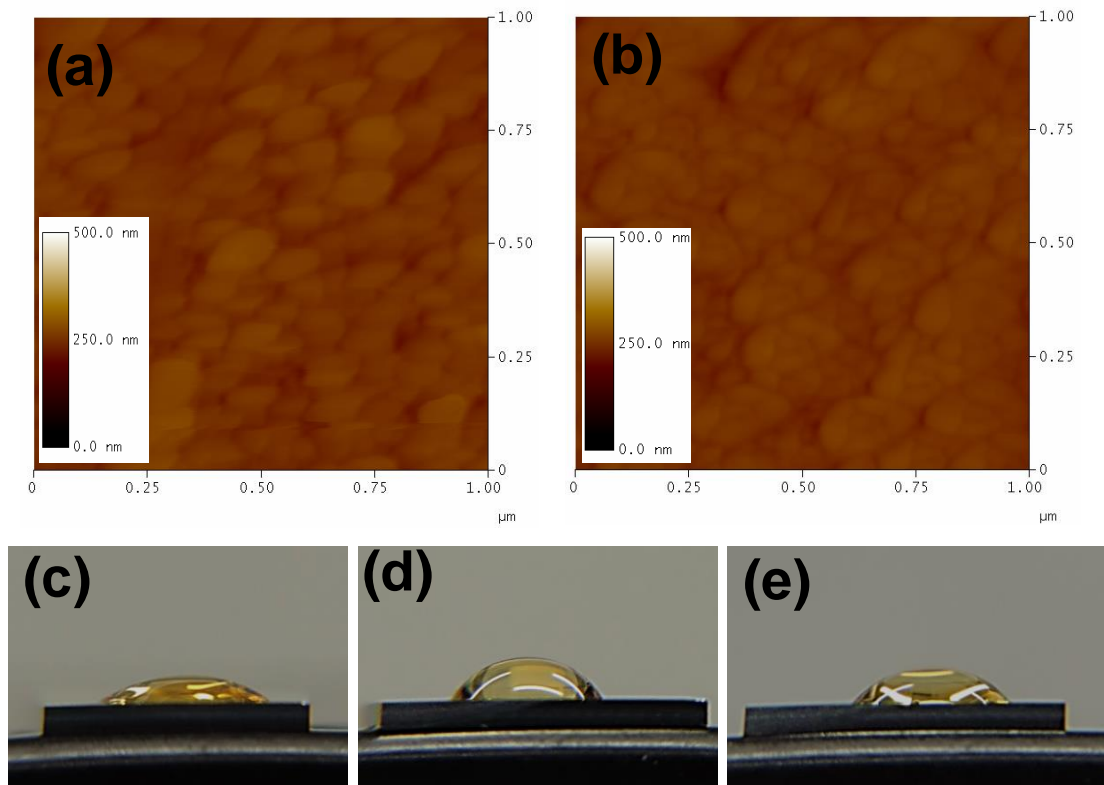


Figure 3 AFM images for (a) blank cleaned gold surface, (b) after anti-TNF- α binding and contact angle images for (c) blank cleaned gold surface, (d) after DTSP SAM formation and (e) after anti-TNF- α binding.

3.3 SB-*EA*-anti-TNF- α /DTSP/Au electrode and its working principle

Figure 4 shows the cyclic voltammetric scans and impedance spectra at every step of the immunosensor electrode modification as indicated in the schematic of Figure 1. In an immunosensor electrode fabrication, DTSP provides the matrix layer on gold and acts as a linker for covalent antibody binding under mild conditions. As seen in Figure 4a, the peak current decreases from 155 μ A for the blank electrode to 105 μ A after DTSP SAM formation, suggesting its layer

formation on the gold surface. This is further confirmed by the increase in charge transfer resistance (R_{ct}) from 141 Ω for the blank chip to 5.18 k Ω after DTSP SAM formation. After binding of anti-TNF- α , a further decrease in peak current to 55 μ A and increase in R_{ct} to 11.90 k Ω suggest successful binding of antibodies on the surface of the DTSP modified gold electrode. A decrease in R_{ct} to 7.04 k Ω and increase in peak current to 70 μ A after EA treatment, indicate the removal of physically adsorbed antibodies on the surface. Finally, after blocking with starting block PBST20, the decrease in peak current to 29 μ A and increase in R_{ct} to 13.70 k Ω suggest successful blocking of free spaces on the sensor surface. Decrease in peak current and increase in R_{ct} after antibody binding and blocking may be attributed to the insulating nature of proteins, which hinder the charge transfer from the solution to the surface of the electrode.

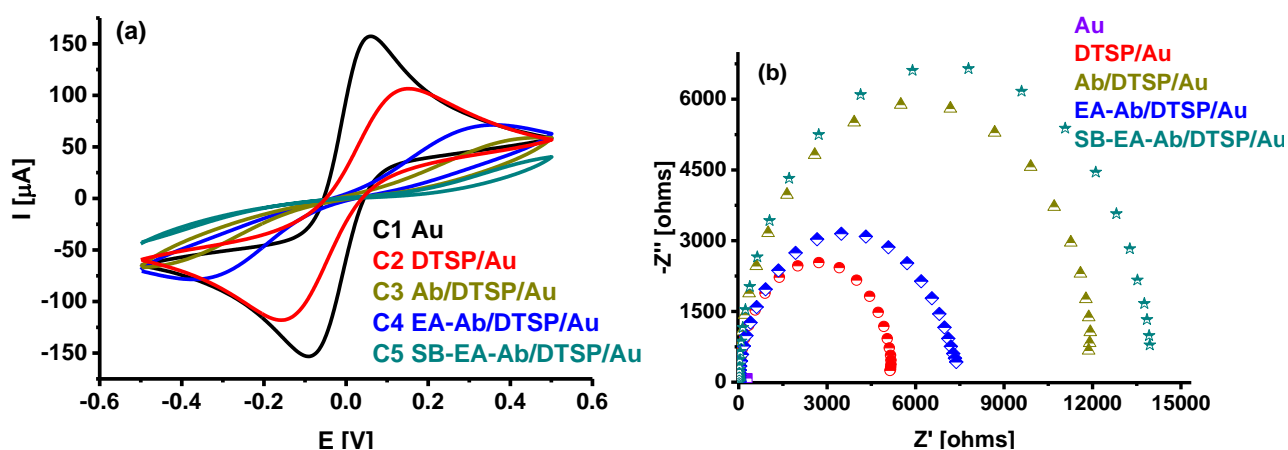


Figure 4. Cyclic voltammetric scans (a) and impedance spectra (b) at every step of the immunosensor electrode modification.

The electroactive surface area of Au, DTSP/Au and EA-Ab/DTSP/Au electrode was estimated via scan rate studies in 5.0 mM $\text{Fe}(\text{CN})_6^{4-/3-}$ with 1 M KCl at different scan rates from 30 to 100 mV/s (see Supplementary Information, Figure S-1 a-c). Oxidation peak current data was then plotted against the square root of the scan rate and the slope was utilized to estimate electroactive surface area using the Randles-Sevcik equation: $I_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} v^{1/2} c$, where I_p is the peak current (A), n is the number of exchanged electrons (1 for $\text{Fe}(\text{CN})_6^{4-/3-}$), c is the concentration of the

electroactive specie (mol/cm^3), A is the electroactive area (cm^2), D is the diffusion coefficient (cm^2/s) and v is the scan rate (V/s). In the studied scan rate range, the oxidation peak currents for Au, DTSP/Au and EA–Ab/DTSP/Au electrodes (see Supplementary Information, Figure S-1 d-f) were found to be linearly dependent on the square root of the scan rate. Using the D value of $0.718 \times 10^{-5} \text{ cm}^2/\text{s}$ for $5 \text{ mM FeCN}_6^{3-/4-}$ in 1 M KCl [25], the active surface area was estimated to be 19.38 mm^2 , 23.02 mm^2 and 21.94 mm^2 for Au, DTSP/Au, and EA–Ab/DTSP/Au, respectively. Observance of such high area might be attributed to radial diffusion occurring on the micro-electrode bands in comb-shaped gold micro-electrode arrays.[26]

3.4 TNF- α response studies

TNF- α interaction with surface bound antibodies was studied at both room temperature and 37°C for different incubation times. It was observed that with increasing incubation temperature to 37°C , the peak current response observed for p-phenol amine oxidation at desired TNF- α concentrations increases; however, the peak current response observed for p-phenol amine oxidation for serum only sample (background signal) is also found to be higher and, thus, not much enhancement in normalized signal (peak current for desired TNF- α concentrations / peak current for serum only) was observed. Similarly, the peak current signal was found to be increasing with increasing incubation time; however, good signal sensitivity was observed at 20 min and no further normalized enhancement was observed for 30 min incubation. Thus, 20 min of incubation time at room temperature (22°C) was selected for further experiments.

Figure 5 (a) shows the DPV spectra for different concentrations ($500 \text{ pg}/\text{ml}$ – $100 \text{ ng}/\text{ml}$) of TNF- α in undiluted serum. The peak current for the immunosensor chips was found to be around $0.145 \text{ }\mu\text{A}$ when tested for serum only and increased from around $0.185 \text{ }\mu\text{A}$ for $500 \text{ pg}/\text{ml}$ TNF- α in undiluted serum to around $6.875 \text{ }\mu\text{A}$ for $100 \text{ ng}/\text{ml}$ TNF- α in undiluted serum. It is clear from the data that with increasing TNF- α concentration in undiluted serum the peak current response

observed for p-phenol amine oxidation increases and the sensor can be used in the tested concentration range. Furthermore, the increasing peak current indicates successful binding of antigens and secondary tags, which resulted in increasing production of the final electroactive product during alkaline phosphatase based catalytic conversion of 4-amino phenyl phosphate to 4-amino phenol, thus producing higher currents for higher concentrations. Figure 5 (b), which shows the spectra at concentrations equal or lower than 5 ng/ml, clearly reveals that the signal for serum is negligible and a good signal is observed even for 500 pg/ml of TNF- α and can easily be differentiated from the background signal of the serum only sample. It is noted that in DPV curves oxidation signal of 4-amino phenol was found to be with a shoulder. To understand the reason for shoulder peak, additional experiments were carried out, where the assay was performed on one chip and tested on blank chip and no such shoulder peak was observed (Supplementary Information, Figure S-2a). However, when the assay was carried out on a surface kept separately on the sensor chip, the 2nd peak at a slightly higher potential was observed (Supplementary Information, Figure S-2b).[27] The exact reason for the shoulder peak is still not clear, however we expect that it arises from the adsorbed molecules on the sensor surface. When biomolecules are covalently attached to the surface, the 2nd peaks moves closer to the peak for p-phenol amine oxidation and the overlapping of the peaks gives rise to a shoulder. Further detailed investigations are currently under investigation.

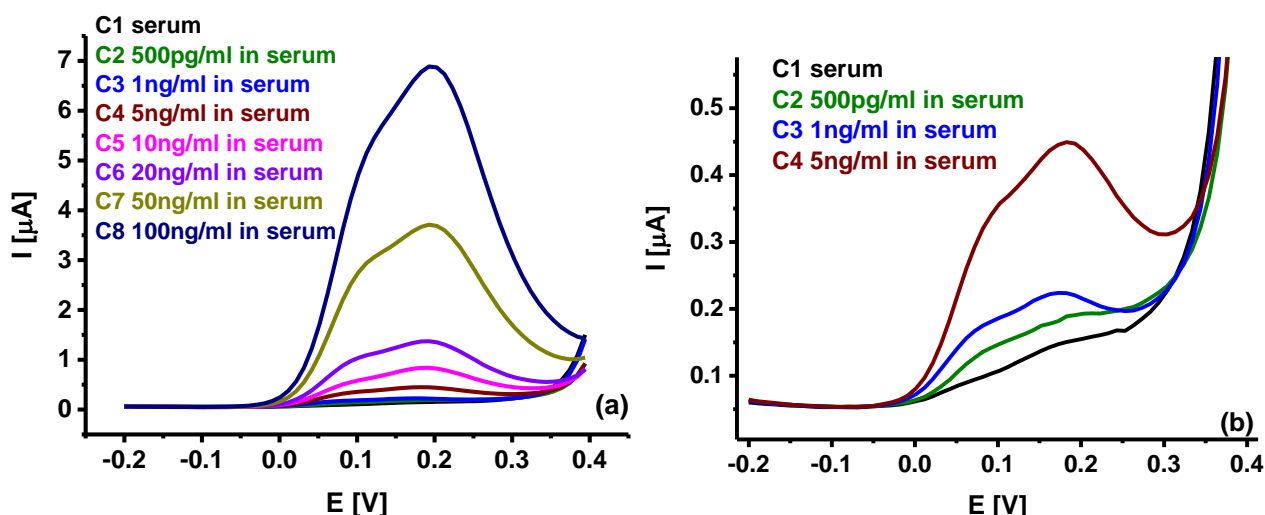


Figure 5. DPV curves for different concentrations: (a) 500 pg/ml to 100 ng/ml; (b) 500 pg/ml to 5 ng/ml of TNF- α in undiluted serum.

Figure 6a shows the normalized peak current at various concentrations of TNF- α . Normalization was done by dividing the peak current for the desired concentration by the peak current observed for serum only. This normalization was done to better show the dose response, as there is a small day to day and batch to batch variation (less than 4%, as indicated by the error bars). It is clear from the dose response that the biosensor chips show linearity for TNF- α sensing in the concentration range 500 pg/ml – 100 ng/ml, which could be characterized using the linear equation: $I_c/I_{c0} = 0.987 + 0.46 c_{\text{TNF-}\alpha} \text{ (ng/ml)}$. The SB-EA-anti-TNF- α /DTSP/Au electrode exhibits a sensitivity of $0.46 \text{ (ng/ml)}^{-1}$ and standard deviation of 0.0092. Detection limit was estimated using $3 \times \text{standard deviation} / \text{sensitivity}$ and found to be 60 pg/ml. Thus, it is clear from the results that the SB-EA-anti-TNF- α /DTSP/Au electrode can be used successfully for testing TNF- α in undiluted serum and can be utilized for developing sensitive biosensors for other proteins as well.

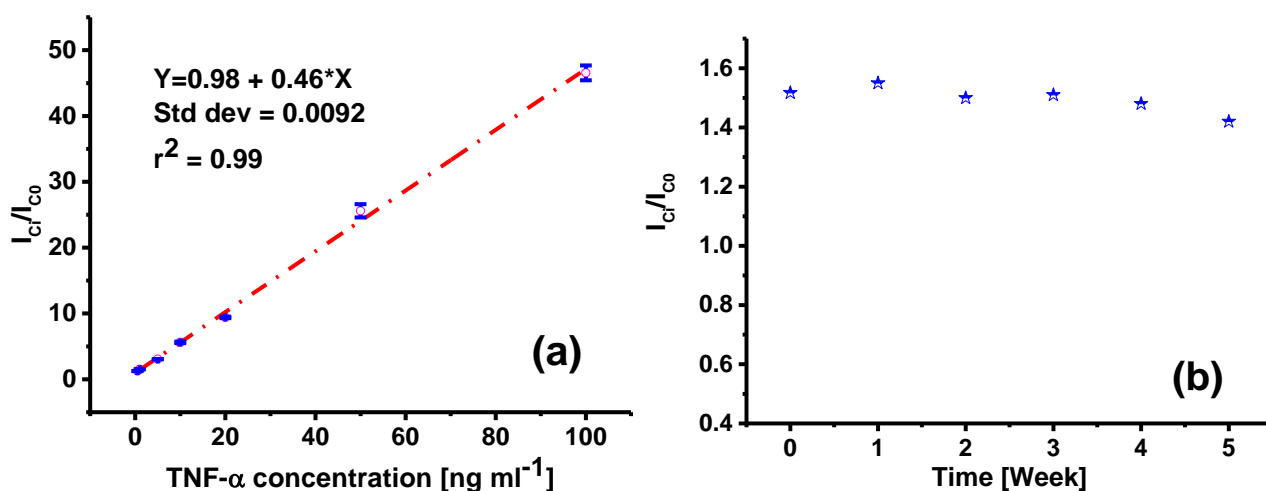


Figure 6. (a) Normalized peak current at various concentrations of TNF- α and (b) normalized peak current for 1 ng ml⁻¹ of TNF- α using SB-EA-anti-TNF- α /DTSP/Au electrodes at various intervals

3.5 Shelf life and selectivity studies

To estimate storage life, DPV studies were performed to test the shelf life of the SB-EA-anti-TNF- α /DTSP/Au electrodes over a 5-week period, at intervals of one week. 1 ng/ml of TNF- α in serum was tested every week on selected electrodes from a set made at the same time and stored at 4 °C when not in use. Figure 6b shows that the SB-EA-anti-TNF- α /DTSP/Au electrodes retain more than 93% of the initial signal even after five weeks, indicating good stability of the biolayer. Also, SB-EA-anti-TNF- α /DTSP/Au electrodes were studied for selectivity studies against other protein markers such as prostate specific antigen (PSA), human epidermal growth factor receptors 2 and 4 (HER2 and HER4) at high concentrations of 100 ng/ml. DPV results of interference study from undiluted serum, 100 ng/ml of PSA in serum, 100 ng/ml of HER 2 in serum and 100 ng/ml of HER 4 in serum did not show significant response to any other protein in serum even at such high concentrations (see Supplementary Information Figure S-3). Thus, the SB-EA-Anti-TNF- α /DTSP/Au electrode offers a new platform for biosensor development to selectively detect protein biomarkers in undiluted serum with high sensitivity. Table 1 compares the results of the present study with other reported results for TNF- α , clearly suggesting that the SB-EA-anti-TNF-

α /DTSP/Au electrode can be used to get better response in a dynamic wide range in undiluted serum samples.

Table 1: Characteristics of the SB–EA–anti-TNF- α /DTSP/Au electrode and those reported in literature.

Immobilization Matrix	Sensing element	Sample medium	Sample incubation time	Detection method	Linearity	Detection limit	Ref.
Gold	MB tagged thiolated TNF- α aptamer	blood	15 min	SWV	10 - 100 ng/ml	10 ng/ml	[15]
DNA nanostructure-decorated gold surfaces	Anti-TNF- α antibody	1% BSA in PBS	60 min	Amperometry	0.1 - 2.5 ng/ml	0.1 ng/ml	[28]
MUA SAM	Anti-TNF- α antibody	Buffer	15 min	QCM	40 – 2000 ng/ml	25 ng/ml	[29]
Avidin-modified SPE	Anti-TNF- α antibody	Buffer	45 min	SWV	0.1 – 1000 ng/ml	0.05 ng/ml	[22]
Gold	MB tagged thiolated TNF- α aptamer	Cell culture medium	30 min	SWV	9 – 88 ng/ml	5.46 ng/ml	[30]
DTSP SAM	Anti-TNF- α antibody	Serum	20 min	DPV	0.5 – 100 ng/ml	0.06 ng/ml	this work

SWV: square wave voltammetry; MUA: mercaptoundecanoic acid; SPE: Screen printed electrode; MB: Methylene blue

4. Conclusion

In conclusion, SB–EA–anti-TNF- α /DTSP/Au electrodes were developed using DTSP SAM as a matrix and immobilizing agent for antibodies. The biosensor shows good selective measurement of TNF- α in undiluted serum in the concentration range of 500 pg/ml – 100 ng/ml, with very low non-specific response to serum proteins. The prepared electrodes showed sensitivity of $0.46 \text{ (ng/ml)}^{-1}$ and a low detection limit of 60 pg/ml. Furthermore, the fabrication method was found be simple and the prepared electrodes found to be stable over a 5 weeks' period when stored at 4 °C in a humid chamber. The studies show that the developed platform is very robust and can be

employed for the detection of other proteins and biomolecules in undiluted serum, paving the way to a range of novel low cost and rapid blood biosensors.

Acknowledgements

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